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PRINCIPAL INVESTIGATOR: Dr. Daniel Medina

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Houston, Texas 77030

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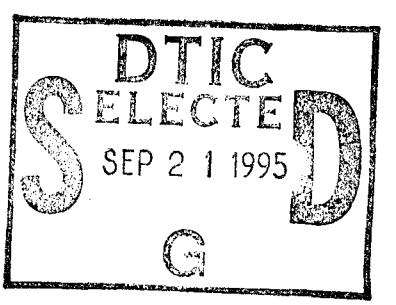

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The goal of this research training program is to produce highly qualified scientists for careers as independent investigators in the field of breast cancer. In the last 20 years, there has been a steady increase in the incidence of breast cancer. During the same period, there has been a fundamental revolution in the understanding of molecular and cell biological concepts related to cell growth, function and tumorigenesis. To utilize what has been learned and to continue future progress in the area of breast cancer requires the continued availability of well-trained, innovative and committed scientists. This program represents an interdepartmental training program involving thirteen investigators from six departments. Trainees are predoctoral fellows with backgrounds in biochemistry, cell and molecular biology, molecular genetics and molecular virology. The training program provides trainees with additional foundation in carcinogenesis and breast cancer. In addition to the core curriculum taken by the predoctoral fellows in their respective academic departments, program enhancement is provided through trainees' participation in a graduate course on "Molecular Carcinogenesis," a short course on "Pathobiology of Human Breast Cancer," a Breast Cancer Seminar Series, and participation at national meetings, and local seminars. Six predoctoral trainees are currently enrolled in the training program.

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Daniel Medina July 13, 1995

PI - Signature Date

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INTRODUCTION:

Breast cancer is a complex disease whose ultimate understanding will require the integration of facts resulting from a multidisciplinary approach. Continued basic science research will provide a fuller understanding of the basic mechanisms of breast cancer which is necessary to conquer the disease in humans. In order to have the scientific human armamentarium to further this understanding, this training grant focuses on producing qualified scientists for careers as independent investigators in the area of breast cancer. The rationale for a targeted training grant in breast cancer is based on the belief that the elucidation of how oncogenes, tumor suppressor genes, hormones and growth factors act at the molecular level and as developmental-specific agents are critical questions directly relevant to the etiology, prevention, diagnosis, treatment and prognosis of human breast cancer. The training program draws together individuals who have an established research and training background in the mammary gland with individuals who have a research and training background in cell biology, molecular endocrinology, molecular biology, molecular virology, viral oncology, molecular genetics and biochemistry. The strength of the program is two-fold. First, the program brings together members of diverse disciplines to focus on the training of predoctoral students for careers in an area which, by its biological nature, is multi-disciplinary. Second, the program brings new intellectual approaches and insights to the problem of breast cancer which will be continued by the next generation of research scientists.

The design of the training program provides for trainees to be exposed to clinical problems and recent advances as well as the multi-disciplinary approaches to answering fundamental questions related to breast cancer research. The familiarity and close proximity of the training faculty facilitate and encourage the development of a new generation of research scientists who will be able to understand the problem of breast cancer at a more complex level and from a multi-disciplinary orientation.

BODY

Six predoctoral students are supported by the training grant. The six students, their departmental affiliation, major advisor, thesis problem and an Abstract of their research is provided below:

a. Annette C. Hollman, Department of Molecular Virology, Dr. Janet S. Butel, "Wnt-1, int-2, MMTVLTR-ORF and p53 cooperatively in mammary tumorigenesis."

The working hypothesis of this research is that different oncogenes mediate discrete steps in mammary tumorigenesis and wild-type p53 counteracts those tumorigenic effects. The aims of this project are the following: (1) To transfect MMECLs of different p53 status with the mammary oncogenes *wnt* -1, *int* - 2, and MMTV-LTR-ORF, singly or in combination; (2) To determine if changes in outgrowth morphology or tumorigenicity correlate with the combination of oncogenes introduced or with the p53 status of the parental cell line; and (3) To determine if p53 mutations occur during the process of tumor formation by cells expressing mammary oncogenes.

During the past year, the status of p53 in the mouse mammary epithelial cell lines (MMECL) EL12 and TM12 have been determined by nucleotide sequence analysis. TM12 cells have wild-type p53 whereas EL12 tumors have a truncated p53 which results in the absence of detectable protein. The amount of p53 protein in TM12 cells is being determined by immunoprecipitation followed by ELC-Western blot, using GST-p53 as a standard for quantitation. The *wnt* -1 and *int* -2 oncogenes will be subcloned into a CMV expression vector. *Wnt* -1, *int* -2, and MMTV-LTR-ORF will be transfected singly into EL12 and TM12 cells.

b. Jeffrey M. Jones, Department of Molecular Virology, Dr. Lawrence A. Donehower, "The role of p53 in mammary tumorigenesis using a mouse model."

The *p53* gene is the most commonly mutated gene in human cancers and as such is often found to be mutated in breast cancer. The p53 protein functions as a tumor suppressor and can act by negatively regulating the cell cycle as well as inducing apoptosis.

The p53 deficient *Wnt-1* transgenic mouse is a model for breast cancer. P53-deficient mice develop tumors at a very early age, however they rarely develop breast tumors. Mice carrying the *Wnt-1* transgene have been well established as a mammary tumorigenesis model. Mice that are both deficient in p53 and that carry the *Wnt-1* transgene develop mammary tumors even more rapidly than their siblings that have a wildtype copy of p53. In addition, mammary tumors that lack the p53 gene show higher levels of genomic instability.

One hypothesis states that p53 may have an additional extracellular role as a tumor suppressor. Cells expressing p53 may be capable inhibiting the abnormal proliferation of adjacent cells. The p53 deficient *Wnt-1* mouse is being used to study the influence of the *p53* status of the cells surrounding the nascent tumor on the rate of tumor growth.

In order to test this idea, cells isolated from mammary tumors of *Wnt-1* transgenic mice of various *p53* genotypes have been transplanted into p53 positive and negative host animals. The rate of tumor formation has been measured. According to the model, tumors that arise in a p53-deficient background will never have been exposed to the putative wildtype p53 associated inhibitory factor. In order for a tumor to grow in a p53 positive environment it must have acquired the mutations necessary to overcome the effects of the inhibitor. Genetic changes in the tumor cells before and after transplantation will be examined to determine whether exposure of p53-deficient cells to a p53-containing environment causes an increase in genetic instability.

This hypothesis is being tested *in vitro* using primary mouse embryo fibroblasts derived from embryos that are either wildtype or deficient for p53. This experiment involves co-culturing MEFs from the two different genotypes. This experiment hopes to demonstrate that the presence of p53 positive cells in a culture is able to inhibit the growth of the p53 deficient cells in that same culture.

c. Sharon Bonnette, Department of Cell Biology, Dr. Daniel Medina, "Mechanism of TGFβ1 inhibition of mammary cell growth."

Transforming growth factor-β1 (TGFβ1) is widely known for its antiproliferative effects on most epithelial cells. The cellular growth advantage of some carcinomas, brain tumors, and melanomas is thought to derive, in part, from the resistance to the antiproliferative effects of TGFβ1. Thus if the responsiveness of the normal or immortal counterpart is compared with the tumorigenic cell type it is frequently noted that the former are growth-inhibition sensitive, whereas the latter are only partially or completely resistant to the antiproliferative effects. A similar finding is observed in two cell lines generated from mouse mammary epithelial cells. An immortal cell line, FSK3, is sensitive to TGFβ1 growth inhibitory effects while its transformed counterpart, responds in a growth stimulatory manner. These two closely related cell lines provide a good system to study the changes in TGFβ1 signaling that occur during tumorigenesis.

An early objective was to rule out whether differences in the levels of TGFβ1 type I, II, and III receptor levels in the two cell lines were responsible for the differential responses to TGFβ1. By ^{125}I- TGFβ1 radiolabeling experiments both cell lines exhibited the same receptor profile which led to the conclusion that the differential response was most likely due to differences in the regulation of downstream molecules.

Downregulation of the synthesis of an early cell cycle molecule, CDK4, has been linked to growth inhibition by TGFβ1 in mink lung epithelial cells. A second objective was to look at the regulation of CDK4 by TGFβ1 in both of these cell lines and see whether differential regulation of CDK4 protein levels was mediating the individual responses. CDK4 synthesis levels in TM3 and FSK3 cells treated with TGFβ1 was examined by methods of ^{35}S methionine metabolic labeling and by western blot. Levels of CDK4 protein did not change in treated vs. nontreated cells. Since regulation of CDK4 is not occurring at the protein level, a third objective is to investigate CDK 4 kinase activity in the cell lines treated with TGFβ1. The aims for the coming year are: First, if kinase activity is affected by TGFβ1 differentially in the two cell lines, changes in cyclin D1 or D2 association with CDK4 and changes in cyclin D total protein levels will be looked at to

see whether changes in this molecule are involved in the regulation of CDK4. Second, the presence of associated CDK4 inhibitors will be investigated. These include p15, p16, p21 and p27. If CDK4 kinase activity is not affected by TGFβ1, CDK2 activity will be assessed in TGFβ1 treated and non-treated FSK3 and TM3 cells to see if this molecule is regulated differentially in the two cell lines and is in part a mediator of the differential responses.

d. Steven Chua, Department of Cell Biology, Dr. Ming-Jer Tsai, "Development of a bitransgenic mouse system to study oncogene function."

The transgenic mice technology has offered a means of targeting oncogenes to the mammary glands to help better understand the role these oncogenes play in the development of breast cancer. In this system, the oncogene is constitutively expressed and studies aimed at discerning critical time points when the mammary glands are most susceptible to oncogenesis remain poorly defined. Thus, it is imperative that a regulatable system be created to allow one to better control the temporal expression of a candidate oncogene to better understand the role this candidate oncogene plays in mammary gland oncogenesis.

An improved bitransgenic mice system is being generated to meet this goal. The first line of mice, the transactivator (regulator) mice, carries a chimeric transcription factor under the control of the MMTV promoter. This chimeric transactivator is made up of an activation domain from HSV-VP16, a Gal4 DNA binding domain and a truncated version of the progesterone receptor ligand binding domain which is responsive to anti-progestins (ie RU486) but not to progestins. The second line of mice, the target mice, carries an oncogene under the control of four 17-mer palidromic Gal4 cis-acting elements (17X4) where the genetically modified transactivator can bind. Two minimal promoters have been chosen for the target genes namely, the E1B TATA and the TK promoter. When these two lines are crossed, in the bitransgenic mice created, the transactivator can then bind to the cis-acting Gal4 sequences upstream of the target oncogene and bring about the expression of the target oncogene when RU486 is administered. The expression of an oncogene is regulated by giving the bitransgenic mice doses of RU486 at specific time points during development and monitoring the effects on the mammary gland. With this system, one can also study the interaction between hormones and the target oncogene in the development of mammary gland. In addition, any possible interaction between NMU and an oncogene or a tumour suppressor can also be elucidated in mammary gland oncogenesis.

The specific aims of this research project are as follows:

1) Determine the role and timing of the polyoma middle T antigen expression in the development of the mammary gland
2) Investigate the interaction between the polyoma middle T antigen and hormones in the development of the mammary gland.
3) Study the interaction between MNU and the polyoma middle T antigen or tumour suppressors in mammary gland development.

The MMTV-GLVP transgenic mice have been generated and three lines currently are being analyzed. The polyoma middle T target construct has been engineering and focus forming

assays demonstrate that the construct is responsive to the regulator. Future experiments will utilize the reagents to examine the role of polyoma middle T antigen in mammary tumorigenesis.

e. Esha A. Gangolli, Department of Cell Biology, Dr. Bert W. O'Malley, "Ligand-independent activation of the human estrogen receptor."

The steroid hormone estrogen is intimately involved in mammary gland development and differentiation and in mammary tumorigenesis. The role of its cognate receptor, the estrogen receptor (ER), in these processes has been extensively studied in an attempt to delineate the precise mechanisms by which estrogen exerts its powerful regulatory and modulatory control. Recently, the ability of ER to be transcriptionally activated in the absence of hormone has added another layer of complexity to this problem. The neurotransmitter dopamine and growth factors such as epidermal growth factor and insulin-like growth factor -I are capable of activating ER in a ligand-independent manner. These agents act via their membrane receptors, presumably through activation of a signal transduction cascade(s). In an attempt to delineate the cellular players involved in this activation, we are studying the activation induced by a dopamine receptor agonist, SKF 82958.

We have utilized a non-recombinant, replication-deficient adenoviral strain (dl312) to efficiently transfect the human neuroblastoma cell line SK-N-SH. An expression vector for the human ER and a reporter construct linking an estrogen responsive element to the chloramphenicol acetyl transferase reporter gene are cotransfected into these cells and tested in a transactivation assay. The full agonist of the D1 subclass of dopamine receptors, SKF 82958 (10 μM), is as efficient as estrogen (1 nM) in eliciting activation of the human ER. This activation is dependent on the presence of a functional ER. This activation is mimicked by 10 μM forskolin and is diminished by over 50% by a 10 μM concentration of the specific protein kinase A blocker H-89. This suggests that protein kinase A may mediate, at least in part, activation by the dopaminergic agonist. We are currently measuring cellular levels of 3',5'-cyclin adenosine monophosphate (cAMP) to confirm this.

f. Deana Roy, Department of Cell Biology, Dr. Jeffrey M. Rosen, "Isolation of molecular markers for terminal end buds in mammary gland development."

Rapid growth and proliferation of TEBs is responsible for ductal elongation. Between 21 and 62 days of age, the TEBs may either differentiate into alveolar buds (AB) or remain as TEBs. Furthermore, at the commencement of estrous (35-42 days of age), a few AB may differentiate into alveolar lobules.

In comparative studies of rat and human mammary tissues, the TEBs of the rat are thought to be equivalent to the intralobular terminal duct of the human. The intralobular terminal ducts consists of a rapidly proliferating population of cells which are extremely susceptible to neoplastic transformation. Studies of carcinogen-induced tumor formation in rats suggest that the cell populations of the TEBs are also targets of neoplastic transformation. When nulliparous rats are treated with the carcinogen DMBA (7,12-dimethylbenz(a)anthracene), the greatest number of

malignant tumors occur when TEBs are present, at 40-50 days of age. This effect is not observed in multiparous rats, in which TEBs no longer exist and alveoli predominate.

The goal of this project is to explore the fate of TEB cell populations during mammary development and carcinogenesis. To do so, genes expressed specifically in the TEBs of the nulliparous rat mammary gland will be isolated, cloned and characterize. These genes will then serve as molecular markers in TEB cell fate studies.

Differential display PCR (DD-PCR) is being used to identify genes which can serve as markers of TEBs. cDNAs, present only in the TEB tissue fraction, have been selected for further characterization. Thus far, 18 TEB specific cDNAs have been identified using DD-PCR. These clones are currently being subcloned, sequenced and identified by homology to GenBank sequences. Additionally, a virgin mammary gland cDNA library is being constructed to isolate full length cDNA clones. In the following year, the specificity of clones to the TEB RNA fractions will be verified by Northern analysis or reverse-transcription PCR.

CONCLUSIONS:

The training program in breast cancer is functioning as planned. Six students are currently enrolled and actively involved in their research phases of their training program. The breast cancer journal club meets every two weeks and it is anticipated that the involvement of additional laboratories will develop with the increased number of research projects on breast cancer due to new funding. This expansion should provide even greater exposure to our students to emerging problems in breast cancer.

REFERENCES:

None

APPENDIX:

None